Aim 1: **Examine the effects of manipulation of the feeding window on female fertility, gestational health, and maternal glycemia during gestation.**

# Background:

### Time-restricted feeding

Time-restricted feeding, meaning a designated and condensed period in which one consumes their daily calories (usually between 6-10 hours in length), is a dietary strategy that is gaining in popular interest (Figure 1). This method is a modality to implement intermittent fasting, which is distinct from two other modalities, alternate day fasting (ADF) where an individual alternates full days of fasting and *ad libitum* feeding, WHATEVER THE 3RD TYPE IS I CAN NEVER REMEMBER. and Studies in humans have demonstrated improvements in insulin sensitivity, hypertension, as well as other molecular markers of health (Halberg et al., 2005; Hatori et al., 2012; Kahleova, Lloren, Mashchak, Hill, & Fraser, 2017; Liu et al., 2019; n.d.; Ravussin, Beyl, Poggiogalle, Hsia, & Peterson, 2019; Sherman et al., 2012; Sutton et al., 2018; Woodie et al., 2018). An overview of rodent models of TRF demonstrate metabolic improvements in insulin resistance without weight loss (Hatori et al., 2012; Liu et al., 2019; Sherman et al., 2012; Woodie et al., 2018). This suggests that time-restricted feeding may be an appropriate strategy for use in insulin resistant pregnant women. To date, no study has evaluated time-restricted feeding of normal chow in mouse pregnancy. Only one study of time restricted feeding during gestation has been published, which utilized 60% high fat diet (HFD) and Wistar rats (Upadhyay et al., 2019). This work demonstrated that HFD-TRF feeding led to similar caloric intake consumed by both HFD-TRF and HFD-AL (*ad libitum*) counterparts, with similar pre-pregnancy body weight gain between these groups. This study did not evaluate body composition, and did not asses maternal insulin sensitivity or glycemic control. For this reason, I propose to study the effect of TRF in mice before and during pregnancy.



Figure 1: Depicts popularity of “intermittent fasting” as a google search term from 2009-2019.

### Pregnancy is a critical time for maternal health and physiological adaptation

Nutrition and nutrient restriction have been well studied in pregnancy. Diet can modulate not only offspring health, but also the health of the mother during, and long after gestation (Walter, 2014; Donnelly, 2019). One such study of maternal food restriction that is largely credited with the burgeoning of the DOHaD field is that of the Dutch Hunger Winter wherein the effects of severe nutrient restriction during pregnancy during extreme rationing in WWII had a profound effect on offspring risk for obesity and cardiovascular disease later in life (Heijmans et al., 2008; Schulz, 2010). Other, less severe instance of food restriction have also been investigated during pregnancy. Another example is Ramadan fasting. Ramadan fasting takes place over the course of a month in the Islam calendar. During Ramadan, all food and water consumption if confined to after sunset and before sunrise. In most cultures who practice Islam, there are two meals, one larger meal after sundown that breaks the fast, and one before sunrise that is smaller. Conception and gestation during Ramadan fasting has been shown to increase the prevalence of low birth weight babies in some (Opaneye, Villegas, & Abdel Azeim, 1990; Ziaee et al., 2010) but not all (Daley et al., 2017; Hızlı et al., 2012) reports.

Pregnancy is a time of profound physiological change for expectant mothers; including the onset of insulin resistance without hyperglycemia and increases in body weight and food intake. The physiological adaptations to pregnancy are thought to maximize nutrient availability for the fetus. This suggests there is a physiological mechanism to reassign the desired glycemic set point, making the study of pregnancy a relevant and important implication for not only overweight and obese women of childbearing age, but also obese adults in general.

### Insulin Resistance

The induction of insulin resistance in the mother during mid and late gestation has evolved to make available extra glucose and free fatty acids in maternal circulation and further prevent maternal storage of these substrates, allowing consistent nutrient flux toward the developing fetus. Although insulin resistance and weight gain are considered normal adaptations to pregnancy, there are many women who experience excessive, pathological insulin resistance and gestational weight gain. Cho and colleagues estimate that globally, gestational diabetes affects 9.8 % of pregnancies in women aged 20-24 years; the prevalence dramatically increases for women of advanced age during pregnancy (45-49 years) to 45.1% (Cho et al., 2018). Furthermore, a meta-analysis of incidence of type 2 diabetes found that women with a history of gestational diabetes are at 7.43 times the risk than women who were normoglycemic during their pregnancies (Bellamy, Casas, Hingorani, & Williams, 2009). This makes insulin resistance during gestation a critical public health problem that deserves research attention. I have shown that pregnant mice have insulin resistance but not hyperglycemia as evaluated by an insulin tolerance test (Figure 2) demonstrating that mice are a tractable system to evaluate pregnancy-associated insulin resistance. This is consistent with previous work on pregnant rodents (S. R. Ladyman, Carter, & Grattan, 2018; Musial et al., 2016).



Figure 2: Pregnancy induced insulin resistance in age-matched pregnant and non-pregnant females.

### Gestational weight gain and food intake

Weight gain is expected for a healthful pregnancy. The Institute of Medicine recommended amount of weight to gain is based on pre-pregnancy body mass index (BMI) (Rasmussen et al., 2010). Since these recommendations were published, many studies have evaluated the prevalence of excessive gestational weight gain. This excessive gain of weight during gestation appears to be highly prevalent, approximately 47% of sampled women (Goldstein et al., 2017). Therefore, the prevalent and problematic excessive weight gain in pregnancy is also an urgent public health problems that needs to be addressed to improve health indices of not only child health, but also maternal cardiometabolic health.

### Digestive efficiency and chrono-disruption

Energy intake is both ingestion of food, as well as the efficiency by which energy is absorbed. Digestive efficiency many change as a function of genotype, physiological state, or diet. Perturbation of the circadian system can lead to preferential absorption of certain macronutrients; such as preferred carbohydrate to protein metabolism and overall increased fatty acid absorption with disruption of Clock done by pan and colleagues(Pan & Hussain, 2009). It has also been demonstrated that timing of food is sufficient to entrain the circadian system (Chaix, Lin, Le, Chang, & Panda, 2019; Sherman et al., 2012).

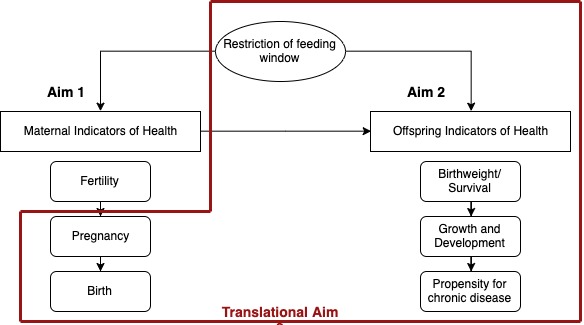


Figure 3: Depicts the specific aims of this dissertation work

Based on the importance of gestational health on the mother and child, and the effectiveness and popularity and effectiveness of time-restricted feeding, it is tempting to speculate that this could be a potential intervention during pregnancy. There are a substantial gaps in our knowledge of the physiology of TRF during pregnancy, and the mechanisms connecting it to maternal glycemia. This chapter will use a rodent model of eTRF to determine effects on maternal fertility, food intake, body composition, and glycemia

## Aim 1.1 Assess the effects of eTRF on female fertility

Fertility represents yet another biological function that demonstrates circadian rhythm (W.-X. Zhang, Chen, & Liu, 2016). As demonstrated by Mereness et al, the coordination of the preovulatory luteinizing hormone surge as well as the coordination of ovary responsiveness to gonadotropins demonstrate entrainment by the CLOCK-BMAL1 system, and even show cell-specific regulation in female mice (Mereness et al., 2016). Swamy and colleagues determined that phase shifting of the period of food availability is sufficient to not only entrain the liver clock in breeding female mice, but also modulate fertility and fecundity(Swamy et al., 2018). Using a light-feeding paradigm, this group demonstrated reduced copulatory plug appearance, reduced embryo implantations, and fewer litters carried compared to dark-fed counterparts (Swamy et al., 2018). Interestingly enough, this was seen without a change in the pattern and regularity of estrous cycling, and furthermore without an increase in post-implantation loss in chrono-disrupted dams (Swamy 2018). The evidence in humans is less clear. A recent meta-analysis of the effects of shift work on infertility demonstrated that the association between shift work and infertility is highly confounded by things like unhealthy lifestyle in shift workers, higher prevalence of risky health behaviors like smoking in shift workers, and the likelihood that women who are working are less likely to have children, creating sampling bias (Fernandez et al., 2016). Furthermore, defining the exposure of shift work makes studies in humans difficult to compare. In general, Fernadez and colleagues suggest that in humans, night shift work is likely to increase complaints of menstrual irregularity, whereas the quality of evidence for shift work and infertility appears to be so inconsistent, there cannot be a statement of risk or of no harm(Fernandez et al., 2016).

The majority of the work done in the circadian biology of fertility has been examined using a chrono-disruption exposure (Salazar et al., 2018; Swamy et al., 2018). While timed feeding cues have demonstrated the ability to entrain peripheral organs and metabolism (Hatori et al., 2012; Pan & Hussain, 2009; Sherman et al., 2012; Swamy et al., 2018), the effects of timed feeding on fertility have not been examined except in models of chrono-disruption.

While this is not the main focus of my dissertation, our study will evaluate whether eTRF prior to pregnancy affects likelihood of conception. Previous reports This will first be done by daily staging of the estrous cycle (Caligioni, 2009) for each dam both before and during the initial exposure to eTRF. Because timed feeding has previously demonstrated the ability to entrain peripheral tissues, including the female reproductive system, I expect that feeding during the shortened period of the dark cycle will not result in irregular or prolonged estrous cycles. Once dams have been mated, daily plug checks will occur until each dam has displayed a copulatory plug. Day of appearance of plug will be coined gestational day 0.5. Days from plug to parturition will be counted. When plug is noted and no litter is born, this will be considered resorption. If resorption occurs, we can furthermore stain collected uteri from dams with 10% ammonium oxide to reveal implantation sites as described by Swamy and colleagues (Swamy et al., 2018). Because one other study has evaluated eTRF in pregnancy, and pups survived to gestational day 18.5, I expect that fertility will not be compromised by eTRF treatment. This is supported by my preliminary data, where eTRF dams had similar rates of pup loss compared to AL controls, and both feeding conditions produced litters.

**Specific aim 1.2 Effects on gestational health of the mother**

**1.2.1 – insulin resistance of pregnancy**

It has been known that insulin resistance occurs progressively in pregnancy in both animals (Musial et al., 2016) and in humans (Sonagra, Biradar, K., & Murthy D.S., 2014). This insulin resistance is related to having available nutrient to shunt toward the growing feto-placental unit.

To compare the effects of eTRF to that of normal pregnancy, an experiment comparing insulin sensitivity in unpregnant females, pregnant AL females, and eTRF females will be conducted. Based on the literature demonstrating more insulin sensitivity in most investigations of TRF in adult subject, I expect that non-pregnant controls will be most insulin sensitive, followed by eTRF dams, and the least insulin sensitive animals will be AL fed dams. Completing this experiment, we will have the first evidence of insulin resistance measures in eTRF pregnancy, but we will also have both a pregnant and non-pregnant control which will provide context to the level of insulin sensitivity.

**1.2.2 – effect of TRF of food intake, body composition, energy expenditure, and digestive efficiency**

Aim 1.2.2.1: Food Intake:  
Food intake increases during pregnancy to allow facilitate sufficient nutrient levels to continue maternal healthful living and to provide energy and essential nutrients for the developing fetuses (S. R. Ladyman et al., 2018). This increase in food intake is usually transient, and most pronounced during the last two weeks of gestation in mice(S. R. Ladyman et al., 2018); followed by a sharp uptick during lactation, with up to 254% more food taken in by lactating mice than age-matched, non- lactating controls(Sharon Rachel Ladyman, Khant Aung, & Grattan, 2018).

One such concern about the use of TRF in gestation is that the narrow eating window would provide too little time to consume sufficient calories to support maternal needs and fetal growth. This is especially a concern based on data available from human trials of TRF. In adult humans, when TRF/IF is employed, there is often a reduction in total kcal intake which then leads to weight loss. However, this is often not seen in animal studies even in studies of HFD feeding with TRF, food intake is similar. Furthermore, Upadhyay and colleagues found that TRF of a high fat diet in gestation yielded offspring growth similar to AL chow fed control pups (Upadhyay et al., 2019). For this reason, I do not expect that dams assigned to eTRF treatment to be unable to consume necessary calories to continue a healthful pregnancy. Preliminary data suggests that with the eating window of 6 hours, there are no differences in total 24-hour energy intake in the preliminary cohort. In our preliminary data, we observe no differences in overall food intake between AL and eTRF mice, even though we detect a 126% increase in energy intake during the restricted window.

Aim 1.2.2.2: Maternal Body Composition

Although only one study has been done in TRF in pregnancy, there have been many studies in non-pregnant adults in humans and in mice that evaluate body weight, body composition, and BMI after treatment with TRF. The literature is divergent in humans and animals. In most studies with humans employing different models of intermittent fasting, there is a moderate reduction of body weight when isocaloric/eucaloric feeding is not employed as part of the study (stote, 2017; Gabel 2018). In rodent models; however, TRF of chow diet usually does not impart weight loss (Liu et al., 2019; Woodie et al., 2018). When High fat diet is given, TRF stimulates body weight loss (Hatori et al., 2012; Liu et al., 2019; Sherman et al., 2012). We will monitor body composition (Fat mass, lean mass, free water) indirectly by EchoMRI and we predict to observe no differences in fat, lean, or free water content compared to gestational-age matched, *ad libitum* fed control. This finding would be especially crucial in the state of pregnancy, as progressive and gradual weight gain is expected and necessary for a successful and healthful pregnancy.

Aim 1.2.2.3 Maternal Energy Expenditure:

Works in TRF of humans and animals have demonstrated mixed results with respect to energy expenditure. In some, energy expenditure is increased using this feeding strategy (Halberg, 2005; Gabel, 2018 ), while more either fail to detect any significant increase in daily energy expenditure (Ravussin et al., 2019) or leave this unexamined (). Based on preliminary results, the food intake and body composition levels are unchanged; however, these are only proxy measurements of actual energy expenditure. It is possible that while food intake and body weight do not have detectable differences, any changes in one of these indices coule be counter-balanced by the other (greater digestive efficiency paired with greater energy expenditure or lower digestive efficiency paired with lower energy expenditure).

Although significant differences in total daily energy expenditure is not often seen, there are often periods where RER is distinct from ad lib controls. Namely, during the night, the RER/RQ lowers, resulting in greater fat utilization, and during the day, a high RER predominates – demonstrating greater metabolic capacity for flexibility in those exposed to TRF. I propose to evaluate maternal food intake in the context of body composition and use those data to calculate feeding efficiency. If feeding efficiency is different between eTRF and AL dams, then I would propose to employ metabolic phenotyping wherein VO2, VCO2, locomotor activity, and food intake would be measured during pregnancy. I hypothesized that feeding efficiency will not be greatly changed between the two groups.

## Aim 1.2.2.4 Maternal digestive efficiency

Although digestion and nutrient utilization are active areas of research in both pregnant animals and humans, the physical and physiological changes of the alimentary canal in pregnancy are not well characterized. The vast majority of work that has been done in both humans and in animals focuses on micronutrient transport and utilization during gestation; especially of iron and calcium(Fisher & Nemeth, 2017; Kovacs, 2000).These studies have demonstrated in animal models that there is hypertrophy of the absorptive surfaces in pregnant animals compared to their non-pregnant counterparts (Sabet Sarvestani, Rahmanifar, & Tamadon, 2015). Still, no measurement of absorptive capacity has been done in the context of 6-hour eTRF on the digestive tract, especially not during pregnancy. This is a critical gap in the literature as circadian rhythms and food restriction have been found to entrain enterocyte nutrient transporters to anticipate caloric intake in animals with intact CLOCK (Pan, 2009). In the case of macronutrient transport, timing of food delivery was found to be a more potent entrainment tool than even light/dark cycle manipulation in mice (Pan & Hussain, 2009). For this reason, I believe that macronutrient and energy absorption will be more complete in dams fed eTRF.

## Specific aim 1.3 Determining how eTRF affects insulin sensitivity and glycemia in pregnant mice

Studies of time restricted feeding have demonstrated improvements in insulin sensitivity in both animals (Hatori et al., 2012; Liu et al., 2019; Sherman et al., 2012; Woodie et al., 2018) and humans (Gabel et al., n.d.; Halberg et al., 2005; Jamshed et al., 2019; Sutton et al., 2018). However, these improvements are usually independent to changes in glycemia (Halberg et al., 2005; Hatori et al., 2012; Liu et al., 2019; Sherman et al., 2012; Sutton et al., 2018; Woodie et al., 2018). In fact, reduction in glycemia was only apparent in 1 human study, and only detectable by the use of continuous glucose monitoring, where they found that night-time glucose was reduced whereas daytime glycemia was unchanged (Jamshed et al., 2019). For this reason, I hypothesize that the use of eTRF in pregnancy will result in greater insulin sensitivity compared to pregnant AL fed animals will be improved and that fasting blood glucose will not be affected. To test insulin sensitivity, an insulin tolerance test (ITT) will be conducted. Previous work has demonstrated that in pregnancy, insulin tolerance is affected, whereas glycemia is not therefore a glucose tolerance test will not be used (Musial et al., 2016). If the ITT demonstrates improved insulin sensitivity, I propose to conduct a hyperinsulinemic-euglycemic clamp during the mid- pregnancy (between E14.5-E17.5), when insulin resistance is known to be greatest in mice (Musial et al., 2016). This will provide more information to further evaluate the mechanisms that underlie insulin sensitivity; such as contribution from hepatic glucose production, peripheral glucose disposal, and whole organ glucose utilization. We will also determine NEFA levels, as fatty acids are a major contributor to gluconeogenesis.

**Specific aim 1.4 Molecular Mechanisms driving effects of eTRF in pregnancy**

After observation of the effects that eTRF will have on fertility, food intake, body composition, and maternal insulin resistance, it will be the goal of this dissertation to detect the molecular drivers of these effects. Because the full characterizing of the effects of eTRF on the mother have not been completed to date, the following mechanistic drivers are based on evidence from scientific literature. As the project progresses, more mechanistic candidates may be explored, while some proposed in this work may fail to explain the effects of eTRF and will therefore no longer be investigated. The specific mechanism of insulin sensitivity after treatment with eTRF in adult humans and animals is incompletely understood. I propose three candidates for a mechanism of action; glucocorticoids (corticosterone), growth and differentiation factor 15 (GDF15), and a reduction in insulinemia.

Glucocorticoids are stress-induced hormones derived from steroids and is released in a diurnal pattern. Glucocorticoids are known to worsen insulin sensitivity, whether from endogenous production (Pivonello, De Leo, Cozzolino, & Colao, 2015) or exogenous administration (Dube, Slama, Basu, Rizza, & Basu, 2015). Corticosterone concentration in the blood increases steadily over rodent pregnancies until late term (Barlow, Morrison, & Sullivan, 1974; Jafari, Mehla, Afrashteh, Kolb, & Mohajerani, 2017). This rise in corticosterone is also known to overlap with the steady rise in insulin resistance of pregnancy in mice (Musial et al., 2016). Therefore, it may be that levels of corticosterone in the circulation could be affected by feeding strategy and may further affect insulin resistance of pregnancy. Cortisol has been responsive to eTRF in humans (Jamshed et al., 2019). Jamshed and colleagues found that morning cortisol was higher in eTRF, and evening cortisol was lower. This could mean that the circadian pattern of cortisol secretion is enhanced by eTRF. This enhanced rhythm of cortisol secretion was seen alongside eTRF individuals having fewer glycemic excursions over 24 hours (Jamshed et al., 2019). To test for circadian entrainment of corticosterone secretion in mice (the predominant glucocorticoid in rodent circulation), we will collect serum from pregnant dams, and non-pregnant controls at both ZT0 and ZT12. This serum will then be tested for corticosterone concentration to understand the relationship between corticosterone and insulin resistance in pregnant mice who have been exposed to eTRF.

Another hormonal candidate mechanism could be growth and differentiation factor, GDF15. The effects of GDF15 are known to be mediated through the GFRAL receptor (Hsu et al., 2017) in the brainstem, and plays a role in weight and appetite regulation (Macia et al., 2012, p. 15; Patel et al., 2019). Furthermore, GDF15 appears to promote ketogenesis and fatty acid catabolism (Hsu et al., 2017), both of which are seen in eTRF (Anson et al., 2003; Jamshed et al., 2019; M. Zhang, Sun, Qian, & Tang, 2018). It is known to increase during gestation, and is associated with reduced food intake, and leanness, and improvements in glucose tolerance (Macia et al., 2012). Sugulle and colleagues demonstrated that GDF15 is elevated in human pregnancies that are complicated by pre-eclampsia and diabetes (Sugulle et al., 2009). It has also been demonstrated that overexpression of GDF15 in adult mice fed both chow or high fat diet was able to reduce glycemic response to IPGTT challenge and had greater insulin sensitivity compared to wildtype controls (Macia et al., 2012). Evaluation of serum levels of GDF15 in eTRF and AL fed dams will be conducted. If meaningful differences in GDF15 are detected in concert with improvements in insulin sensitivity, future experiments may investigate the effects of eTRF in GDF15 knockout mice.

One of the few fairly consistent findings of time-restricted feeding trials in both humans and animals is a reduction in insulinemia. Insulin concentration increases are known to be a part of the natural history of type II diabetes disease progression. To test the role of insulin in the effects of eTRF, serum insulin will be measured in both eTRF and AL dams and compared. I hypothesize that insulin secretion will be reduced in eTRF dams, and therefore insulin sensitivity will be increased. The tissues specific-mechanisms of insulin signaling can also be investigated.

## Methods:

### Animals:

C57BL6/J mice were previously used in the insulin resistance of pregnancy experiment were used in this experiment. At 134 days of age, age matched females were randomized to either *ad libitum* (AL) or early time-restricted eating (eTRF). Dams randomized to AL feeding had 24-hour access to chow (5% fat, 24% protein, 3.7% sucrose, 32% starch). Dams randomized to eTRF feeding were allowed *ad libitum* access to chow during 6 hours of the dark cycle (8pm-2am). At 2 am, all dams were moved to clean cages to standardize stress and handling between feeding regimens (Hatori, 2012). Animals were held in a 12:12 light dark cycle, in a temperature and humidity-controlled facility. Food intake was monitored daily, with 6 hour and 24-hour intake calculated as total grams of food consumed per day multiplied by utilizable energy in the provided diet. Dams were switched to AL feeding upon parturition.

### Mating:

Dams were singly housed for the course of the experiment. After a one-week acclimation period, males were added to the cages in monogamous pairs. Males were allowed to remain in cages until copulatory plug appeared, which was noted as day 0.5 of pregnancy. At gestation day 19, males were removed to prevent second pregnancies after delivering. During the course of the birth and post-natal period until PND 21, dams were singly housed with their litters.

### Body Composition:

Once a week, Dams weight was measured weekly using an electronic scale (Mettler Toledo). Body composition including fat mass, lean mass, and free water was assessed indirectly via magnetic resonance imaging (EchoMRI).

### Insulin Sensitivity:

*Insulin tolerance test:*

Insulin sensitivity was assessed by insulin tolerance test 16 days after mating began. Gestational age during ITT was determined using plug data, body weight gain, and date of delivery. As a result, most dams were in the 1st or 3rd week of gestation during this time. After 6-hour fast, blood glucose was taken using a glucometer and tail clip. Females were given insulin injections (0.75 units/kg body weight; Humulin U100 in cold sterile, filtered Phosphate buffered saline (PBS)) and blood glucose was tested using a glucometer at 15-minute intervals for 2 hours. If animals began to exhibit moribund behaviors, 300 units of 10% glucose in cold sterile filtered PBS was administered and subsequent BG measurements were omitted from the ITT.

*Hyperinsulinemic-euglycemic clamp:*

After mating and confirmation of pregnancy by weight gain of 1.75g signaling 7 days of pregnancy (Heyne et al., 2015), animals will be placed singly housed into a special cage unit. Dams will be cannulated and exogenous insulin will be administered, inducing a state of hyperinsulinemia (Kim, 2009) and thereby suppressing hepatic glucose production. Glucose is infused to maintain blood sugar, with the amount infused correlating to the ability of that animal to utilize insulin to dispose of glucose. Greater glucose infusion rates represent more insulin sensitive animals. This method also allows for understanding of tissue-specific glucose disposal through the use of radiolabeled glucose.

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### Glycemia:

Fasting blood glucose will be assessed with a tail vein blood collection and a glucometer immediately preceding the insulin tolerance test. However, as Jamshed and colleagues have previously demonstrated, the use of continuous glucose monitoring may demonstrate more significant trends in glycemia that static blood glucose and terminal blood glucose measurement are able to capture (Jamshed et al., 2019). For this reason, if no clinically significant differences in glycemia between dams arises, I propose to use continuous glucose telemetry during pregnancy to collect 24-hours of continuous glucose measurements without the need for serial sampling and reduction in maternal blood volume. With the collaboration and expertise provided from the animal phenotyping core, implantable glucose telemetry units will be implanted into dams during early pregnancy. The telemetry units collect glucose data can collect data anywhere from 28 to 45 days; therefore, glycemia during the entire pregnancy can be captured with this implantable device.

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### Energy Expenditure:

CLAMS

### Digestive Physiology:

*Energy Absorption*

To determine if there exist any differences in the amounts of energy consumed from food consumed between eTRF dams and ad libitum fed dams, fecal calorimetry will be performed. Full 24-hour fecal samples will be collected from dams individually and then dried. Dried fecal matter will be assessed by a bomb calorimeter to determine total energy content in the stool as described by Murphy and colleagues (Murphy et al., 2010). Results will be expressed as total energy intake for that day – energy found in stool.

*Macronutrient absorption into portal circulation*

macronutrient absorption should be assessed in vivo. This can be accomplished through the use of *in situ* looping of the intestinal tract. Anaesthetized dams at day 17.5 will have two small incisions made on the abdomen, and peritoneal cavity will be flushed with PBS. A proximal jejeunal loop will be made and a mixture of PBS/radiolabeled macronutrient solution (PBS/ [3H] Triolein/[14C] Cholesterol and cholesterol for lipid absorption, and [14C] alpha Methylglucoside (αMG) for carbohydrates, and [3H]glycylsarcosine (gly-sar) for protein) will be introduced to the lumen of the loop via microsyringe. After 1-hour elapses, loops will be collected as well as blood samples from the portal vein. Blood will then be centrifuged at 4 degrees C at 5000 RCF for 20 minutes to collect serum. Serum will be analyzed by scintillation counter to quantify nutrient absorption into portal circulation.

**Potential Pitfalls and Alternative Approaches**

Fertility-specific pitfalls

Due to the restrictive nature of this feeding regimen, we may observe reduced litter size or litter numbers in eTRF fed dams. If this becomes apparent, further examining the reason for reduced pups and litters will be of great importance. It could be that the reduction in litters is behavioral, in that there is reduced mating drive in mating pairs exposed to eTRF. Swamy and colleagues noted that implantations were not reduced in chrono-disrupted mating pairs, but fewer plugs and litters resulted in their union. To test if the problem is implantation related – staining of dams’ uteri post mortum for implantations will be critical. If implantations largely correllates with number of pups produced per dam, it will be necessary to observe and characterize mating behavior and frequency.

Maternal nurturing behavioral pitfalls

Maternal behavior is a critical modulator in both maternal and offspring health. In mice, it is known that there is a risk of cannibalism of offspring(Weber, Algers, Würbel, Hultgren, & Olsson, 2013). Unfortunately, this effect is largely unavoidable, in that C57BL/6J mice are known to partake in this behavior. In order to minimize this effect, handling of pups for counting and weighing will be minimal and animals who are not nulliparous will be used as breeders, in the hopes of maternal instinct being more developed after having had one litter before mating under eTRF or AL conditions.

Null Findings

Although there is an anticipated insulin-sensitive and normal body composition phenotype, it may be that eTRF fails to impart any effect on fertility, feeding, body composition, and maternal insulin sensitivity. If this is found, it will still be of great public health importance, as there would be no preliminary evidence to suggest harm for mother or child when the dyad observes this feeding paradigm. This would require further experimentation and phenotyping to confirm.

Circadian rhythm of hormones and metabolism

As much of metabolism and humoral release is coordinated by the circadian clock system, the timing of samples must be considered. Because continuous sampling for blood is impractical and create hypovolemic stress for both the dam and the gestating offspring, beginning the sampling for hormones, glucose, and other candidates must first be undertaken as static measures. If there is a circadian pattern to the effect of eTRF, like there is known to be for cortisol, a morning and evening sample should be compared. If the diurnal relationship for these indices is unclear with two samples, then continuous sampling may need to be employed. The use of glucose or blood pressure telemetry can help to give a more accurate picture of the circadian rhythm of hormone and metabolism. However, as these methodologies are costly and may exert additional stress on recently impregnated dam, this method will be employed only if necessary to clarify the effects of eTRF.